

The Biological Role of Insulin-like Growth Factor Binding Protein-2 in Tumorigenesis and Metabolic Homeostasis

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Abstract

The Insulin-Like Growth Factor (IGF) system is involved in the regulation of cell growth, differentiation and sustains survival in several tissues. The Insulin-Like Growth Factor Binding Proteins (IGFBPs) are a family of six proteins with high-affinity for IGFs that comprise an important part of the IGF system. IGFBPs have been demonstrated to modulate IGF bioactivity in a spatiotemporal way. This effect on IGF actions can both be stimulatory and inhibitory. More recently, IGF-independent effects of IGFBPs have been revealed that play important roles in tumor cell growth and progression. IGFBP-2 is the second most abundant IGFBP in circulation and its expression pattern has been shown to be altered in many pathological conditions. This review focuses on the biological role of IGFBP-2 in tumorigenesis and metabolic homeostasis.

Chapter 1: The IGF system

1.1 Overview of the IGF system

The IGF family consists of two growth promoting peptides called IGF-1 and IGF-2, two types of IGF receptors (IGF-1R and IGF2-R), and six high-affinity IGFBPs (IGFBP-1 to IGFBP-6). These peptide hormones, cell surface receptors and circulating factors comprise a complex system that plays an important role in the regulation of cell growth, survival and differentiation in several tissues [Chesik et al., 2007].

IGF-1 is an important trophic factor in circulation and stimulates DNA synthesis and cell growth. Furthermore, IGF-1 can inhibit apoptosis in various cell types. IGF-1 actions depend on developmental stage, microenvironmental conditions, tissue specific properties and interaction with other growth factors. Developmental roles of IGF-1 are neuronal differentiation, survival and myelinogenesis [Russo et al., 2005; Chesik et al., 2007]. During adult life, IGF-1 has a neurotrophic and neuroprotective role and IGF-1 is therefore mainly expressed in neuronal rich areas of the brain such as the hippocampus, midbrain and cerebral cortex. The hippocampus and prefrontal cortex, which are essential for cognitive functions, contain high numbers of IGF-1 receptors. In the elderly, a strong correlation is found between the reduction in circulating IGF-1 levels and a decline in cognitive functions [Van Dam et al., 2004].

Although most organs and cell types may synthesize IGF-1, circulating IGF-1 is predominately produced by the liver. Growth hormone (GH) is the major regulator of IGF-1 synthesis but it has no effect on IGF-1 levels in the brain [Chesik et al., 2007; Russo et al., 2005]. IGF-2 is structurally related to IGF-1 and is the most common IGF in the CNS where it is mainly expressed in myelin tissue [Chesik et al., 2007]. It has been demonstrated that both IGF-1 and IGF-2 are essential for normal prenatal growth. Mice with targeted inactivating mutations of either IGF-1 or IGF-2 are born at approximately 50% and 60% of normal body weight, respectively. An increase in IGF levels resulted in increased growth, often in an organ-specific manner [Pintar et al., 1996].

Under normal circumstances, by far most of the IGFs (> 99 %) in the circulation are firmly bound to IGFBPs with higher affinities than that of IGFs for the IGF-I receptor. IGFBPs are secretory proteins that are produced by many if not all tissues and are involved in the regulation of the biological activities of the IGFs [Rajaram et al., 1997; Kuang et al., 2006]. The biological activity of IGFs is determined by the amount of unbound, free IGF that is locally available for binding to IGF-1 receptors. The IGFBPs have common structural and functional characteristics and are believed to originate from early vertebrate evolution [Firth et al., 2002]. Although there is an 80% sequence homology between the genes of the six IGFBPs, each IGFBP exhibits unique properties and functions and is expressed in a tissue dependent manner [Chesik et al., 2007; Ferry et al., 1999]. Three domains can be recognized in IGFBPs: a conserved N-terminal domain, a highly variable midregion and the conserved C-terminal domain [Hwa et al., 1999; Kuang et al., 2006]. In healthy adult subjects, more than 80 % of the IGFs are present in 150-kilodalton (kDa) ternary complexes with IGFBP-3 or IGFBP-5 and an acid-labile subunit (ALS) that cannot cross the capillary barriers. The ALS increases the molecular mass of IGF/IGFBP-3 to limit the access of IGF to the extracellular fluid and tissues [Rajaram et al., 1997]. The half-life of IGFs in the ternary structure is of a magnitude longer than that of free IGF-1. A relatively small proportion of the serum IGF pool

is carried by IGFBP-3 and other IGFBPs as 40-50 kD binary complexes. Less than 1% of IGFs circulates in an unbound form [Ferry et al., 1999; Kelley et al., 1996]. IGFs associated with binary complexes and the free fraction of IGFs are readily exchangeable with tissue compartments [Firth et al., 2002]. Hence, turnover rates in circulation of these IGF pools ($T_{1/2}$: 20-90 and ~10 min, respectively) are much higher than that of the 150 kD complexes (~12 hr) [Rajaram et al., 1997].

Classically, it was thought that IGFBPs exert their actions only indirectly by coordinating and regulating IGF bioavailability in several ways: 1) to regulate transport of IGFs in plasma and between intra- and extravascular spaces, 2) to prolong the half-life of IGFs (150 kD complexes) and regulate clearance, 3) to provide tissue and cell type specific binding sites for IGFs, and 4) to stimulate, inhibit or modulate interaction of IGFs with their receptors [Ferry et al., 1999; Russo et al., 2005; Chesik et al., 2007]. Local regulation of the bioavailability of IGFs is accomplished by mechanisms that alter the affinity of IGFBPs for IGFs such as phosphorylation, proteolysis by specific IGFBP proteases that cleave IGFBPs, thereby forming fragments with reduced or no binding affinity for IGFs, and cell surface association [Rajaram et al., 1997; Ferry et al., 1999; Schneider et al., 2000; Russo et al., 2005].

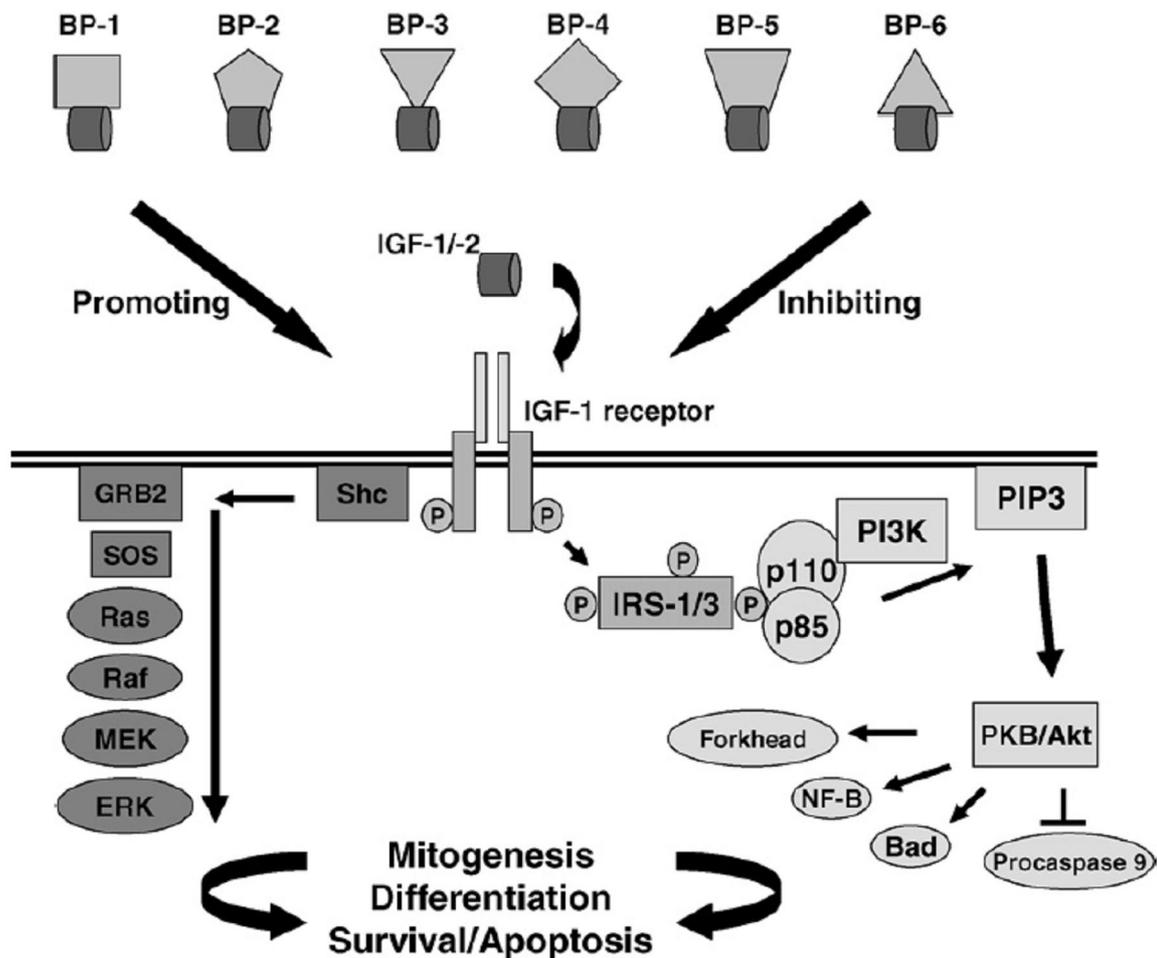


Figure 1. The IGF system. The IGF family is comprised of two growth promoting peptides, IGF-1 and IGF-2, type 1 and 2 IGF receptors, and the six known high-affinity IGF binding proteins. IGF binding to the IGF-1R leads to the activation of two main downstream signaling cascades, the MAPK and PI3K cascades. These cascades are involved in the regulation of cell growth, differentiation and survival in many tissues. (Source: Chesik et al., 2007)

Recently, it appeared that IGFBPs also exert direct, i.e. IGF-independent actions which will be discussed in more detail later. The levels and effects of IGFBPs and IGFs are modulated by specific IGFBP proteases that cleave IGFBPs, thereby forming fragments with reduced or no binding affinity for IGFs [Russo et al., 2005; Ferry et al., 1999].

IGF-1 and IGF-2 actions are mediated by the specific cell surface receptor IGF-1R which is homologous to the insulin receptor. IGF binding to the IGF-1R activates two intracellular signaling cascades, the Mitogen-Activated Protein Kinase (MAPK) and Phosphatidylinositol 3-Kinase (PI3K) pathways which ultimately lead to mitogenesis, differentiation and cell survival (Fig.1) [Russo et al., 2005; Chesik et al., 2007; Samani et al., 2007]. Growth-promoting effects of both IGF-1 and IGF-2 are mediated through the IGF-1R. In addition, IGF-2 also exerts mitogenic effects through high-affinity binding to isoform A of the insulin receptor [Frasca et al., 1999]. The IGF-2R, which is identical to the cation-independent mannose-6-phosphate receptor, is not thought to be involved in cell signaling and has no known growth functions [Kelley et al., 1996]. Researchers believe that the IGF-2R is involved in targeting IGF-2 for degradation by providing a degradative pathway via receptor-mediated endocytosis, thereby inhibiting IGF-2 interaction with IGF-1R [Firth et al., 2002; Chesik et al., 2007]. IGF-2R gene disruption in mice resulted in an increase in fetal size. This is probably because IGF-2 is not targeted for degradation. These IGF-2R null mice display a 5-fold increase in fetal IGFBP levels [Pintar et al., 1996].

In this thesis we will focus on the biological role of IGFBP-2 in tumorigenesis and metabolic homeostasis. From previous research it appeared that IGFBP-2 is an interesting protein for further study because IGFBP-2 has an altered expression pattern in many malignancies and recently it appeared that IGFBP-2 also exhibits direct, IGF-independent actions.

Chapter 2: IGFBP-2

2.1 Structural characteristics and expression of IGFBP-2

The IGFBP-2 gene is located on the long arm of chromosome 2 region q33-q34 near the IGFBP-5 gene and is encoded by four exons [Fukushima et al., 2007]. The N-terminal domain is encoded within exon 1, the midregion within exon 2 and the C-terminal domain within exon 3 and 4 [Hwa et al., 1999; Ferry et al., 1999]. The N-terminal domain contains six disulfide bonds and the C-terminal domain contains three disulfide bonds. Both the N-terminal and C-terminal domain are cysteine-rich and involved in high-affinity IGF binding [Murphy, 1998; Carrick et al., 2001; Firth et al., 2002; Kibbey et al., 2006]. In more detail, the N-terminal domain is needed for rapid association of IGFBP-2 with IGF and the C-terminal domain makes sure that IGFBP-2 and IGF maintain a complexed form [Carrick et al., 2001]. The midregion of IGFBP-2 does not bind IGFs but is needed for an optimal relationship between the N-terminal and C-terminal domains [Hwa et al., 1999]. Another important property of the midregion is that it contains four major proteolytic cleavage sites. Cleavage by proteases results in the loss of IGF binding affinity and can therefore regulate IGF activity [Clemmons, 1997; Fukushima et al., 2007].

IGFBP-2 contains a heparin binding domain and an Arg-Gly-Asp (RGD) sequence indicating an integrin binding motif. This allows IGFBP-2 to interact with cell surface integrin receptors and extracellular matrix (ECM) components such as vitronectin, laminin, collagens and fibronectin [Firth et al., 2002; Chesik et al., 2007]. All these (glyco)proteins act as cell adhesion and signaling receptors that can regulate proliferation, migration, tissue remodeling and cell death [Pereira et al., 2004; Hood et al., 2002]. IGF/IGFBP-2 complexes have a higher affinity with the ECM than unbound IGFBP-2 has, and are more susceptible to protease-mediated IGFBP cleavage. Membrane association results in a lower IGFBP-2 affinity for IGFs, thereby initiating the release of IGF. This may concentrate IGFs close to their receptors and increases their mitogenic potential [Chesik et al., 2007; Clemmons, 1997].

IGFBP-2 is the second most abundant IGFBP in the circulation, after IGFBP-3, and has a preferential affinity for IGF-2 over IGF-1 [Hoeflich et al., 2001; Wolf et al., 2000]. It is a nonglycosylated protein of 31 kDa and contains 289 amino acids [Rajaram et al., 1997; Kelley et al., 1996]. This mature protein arises from precursor IGFBP-2 that has a molecular weight of 36 kDa and consists of a signal peptide together with the three distinct domains [Fukushima et al., 2007]. Mature IGFBP-2 forms a binary complex with IGF-1 or IGF-2 with a molecular weight estimate of 40-50 kDa. IGFBP-2 in circulation originates from hepatocytes and regulation of IGFBP-2 gene expression in plasma seems complex because several hormones and growth factors can influence this diversely. Reported hormones that are involved in regulating IGFBP-2 expression include GH, IGF-1, IGF-2, insulin, interleukin-1, estrogen, glucocorticoids, transforming growth factor- β (TGF- β), human chorionic gonadotropin and follicle-stimulating hormone (FSH). However, signaling pathways that are involved remain essentially unclear [Boni-Schnetzler et al., 1990; Hoeflich et al., 2001; Martin et al., 2007; Huynh et al., 2008]. IGF-1 and insulin stimulate IGFBP-2 synthesis in a human embryonic kidney cell line [Boisclair et al., 1994]. IGF-2 has been shown to be involved in regulating IGFBP-2 expression because elevated circulating IGFBP-2 levels are found under various conditions where the free IGF-2 level in serum is expected to be elevated [Blum et

al., 1993; van Doorn et al., 2001]. Furthermore, enhanced serum IGFBP-2 levels are found in transgenic mice that overexpress IGF-2 [Wolf et al., 1994]. Martin et al. [2007] wanted to identify pathways and regulatory factors that are involved in the expression of IGFBP-2 in breast cancers. Therefore, they used the MCF-7 breast cancer cell line as a model. A marked decrease in IGFBP-2 expression was seen when MCF-7 cells were exposed to inhibitors of PI3K and MAPK signaling pathways (Fig. 1), which are dysregulated in many cancers. The same effect was seen if the PI3K pathway was blocked further downstream by rapamycin. The PI3K signaling pathway can be stimulated by IGF-1 which also increases IGFBP-2 expression. Activation of IGF-1R by IGFs stimulated IGFBP-2 expression in MCF-7 cells and this was also due to the PI3K pathway. However, results indicated that PI3K pathway activation was necessary but not sufficient for IGFBP-2 expression so other factors must be involved. Estrogen regulation of IGFBP-2 expression has been reported in breast tumors [Milewicz et al., 2005] and other cell types [Cardona-Gomez et al., 2000] and therefore Martin et al. tested whether IGFBP-2 expression of the MCF-7 cells was estrogen sensitive. It turned out that estradiol, the major estrogen in humans, synergizes with IGF-1 to stimulate IGFBP-2 expression. IGFBP-2 expression by IGF and estradiol stimulation can be blocked by inhibition of either the PI3K pathway or IGF-1R [Martin et al., 2007].

Expression of IGFBP-2 has been demonstrated in various tissues and fluids but it is the major IGFBP in cerebral spinal fluid due to its production by multiple neural tissues [Ferry et al., 1999]. Expression of IGFBP-2 can already be detected early in embryogenesis, especially in the CNS. In particular, brain regions that undergo continuous remodeling such as the hippocampus and cerebellum [Russo et al., 2005]. However, IGFBP-2 is also predominantly expressed in the liver, adipocytes and reproductive system [Wheatcroft et al., 2009].

IGFBP-2 and IGFBP-1 are the most abundant binding proteins during fetal development but levels decline during the early neonatal period and IGFBP-3 becomes predominant [Rajaram et al., 1997]. Senescence and low levels of serum IGFBP-2 are seen during puberty but these levels steadily increase with age and this increase accelerates at age 60 and above which indicates age-dependency [Rajaram et al., 1997; Mattsson et al., 2008].

The levels of most of the IGF system components change with age and this could contribute to an impairment in function of several organs during ageing. In general, an overproduction of inhibitory IGF system components together with an underproduction of stimulatory IGF system components can be detected (Table 1) [Rajaram et al., 1997].

IGF system component	Puberty	Ageing
IGF-1	Increase	Decrease
IGF-2	Increase	Decrease
IGFBP-1	Decrease	Increase
IGFBP-2	No change	Increase
IGFBP-3	Increase	Decrease
IGFBP-4	No change	Increase or no change
IGFBP-5	Increase	Decrease
IGFBP-6	Increase	Increase

Table 1. IGF system components and the change in serum levels during puberty and ageing. (Source: Rajaram et al., 1997; van Doorn et al., 1999; van Doorn et al., 2001)

Serum IGFBP-2 levels are relatively stable and are not influenced by changes after eating a meal. This may be due to the long half-life of IGFBP-2 compared to other IGFBPs [Murphy,

1998]. However, IGFBP-2 concentrations are metabolically regulated because a major increase in IGFBP-2 level can be seen in people on protein restriction. Examples are people who are fasting for more than a week or patients with anorexia nervosa. Normalized serum IGFBP-2 levels are established after protein refeeding but full normalization requires high-protein intake [Rajaram et al., 1997; Chesik et al., 2007]. Also, administration of GH, which is known to decrease plasma IGFBP-2 in animals where the pituitary gland was removed, did not result in a change in protein restricted adults [Clemmons et al., 1991]. Pathological conditions that increase serum IGFBP-2 levels include non-islet-cell tumor hypoglycemia, diabetes mellitus, chronic renal failure, liver cirrhosis and certain types of leukemia [Wolf et al., 2000; Chesik et al., 2007].

It has been reported that cellular actions of IGFBP-2 on IGF-mediated functions can be both inhibitory and stimulatory. A spatiotemporal relationship of IGFBP-2 with IGF-1 has been demonstrated during cerebellar development. Expression of IGFBP-2 and IGF-1 levels peak at the same time during postnatal maturation which suggests a close relation in the developing CNS. Other studies demonstrated that IGFBP-2 is involved in IGF-1 transportation. IGF-1 levels decline after development of the CNS, except in brain regions that undergo continual neural renewal and differentiation [Chesik et al., 2007].

Spatiotemporal modulation of IGF-2 activity is regulated by IGFBP-2 in the CNS. It has been demonstrated that IGF-2 production often is associated with IGFBP-2 expression. Serum levels of free IGF-2 seem to be the major regulator of IGFBP-2 in circulation which is also demonstrated in transgenic mice overexpressing IGF-2. These mice display increased levels of serum IGFBP-2 which will be explained in more detail later [Wolf et al., 1994; Chesik et al., 2007]. It has been suggested that IGFBP-2 plays a role in transporting IGF-2 and protects it from proteolytic degradation. IGFBP-2 may also be responsible for preventing IGF-2 interaction with the IGF-2R, thereby leaving IGF-2 available for IGF-1Rs [Russo et al., 2005; Chesik et al., 2007].

2.2 Phenotypic alterations of IGFBP-2 null mutant mice

To study the effect of IGFBP-2 during development, a mouse model was created in which the mice lack most of exon 3 of the IGFBP-2 gene. Homozygous IGFBP-2 mutant mice do not contain IGFBP-2 messenger ribonucleic acid (mRNA) and protein but are still viable and fertile [Wood et al., 2000].

No difference in birth weights was found between wild-type and IGFBP-2 knockout mice suggesting that prenatal growth was not affected by IGFBP-2. After birth, the mice were weighed until day 35 to find out if the IGFBP-2 mutation affects postnatal growth. Again, no differences in body weights were found between both groups suggesting that postnatal growth is also not affected by the mutation [Wood et al., 2000; Schneider et al., 2000]. Next, several organs, including heart, liver, kidneys, lungs and spleen, were weighed to determine whether the IGFBP-2 mutation caused changes in organ growth. Only the spleen of homozygous IGFBP-2 mutant mice weighed significantly less (25-30%) than that of wild-type mice whereas the liver weighed significantly more (~15 %). The reason for a reduction in spleen weight could be either a decrease in transport of IGF-1 to the spleen or a decrease in autocrine/paracrine actions of IGF-1 in the spleen itself. The increase in liver size may also be due to autocrine/paracrine actions of IGFBP-2. However, it is unclear why the absence of

IGFBP-2 leads to opposite effects in spleen and liver [Wood et al., 2000; Schneider et al., 2000].

A possible explanation for the small differences between wild-type mice and IGFBP-2 null mutants is that other IGFBPs compensate for the absence of IGFBP-2. Indeed, an increase in circulating levels of IGFBP-1, IGFBP-3 and IGFBP-4 levels was found in the homozygous IGFBP-2 mutant mice, indicating that other IGFBPs are able to compensate for the absence of IGFBP-2. IGFBP-2 is already expressed during embryonic development in several tissues but only small phenotypic alterations could be found which also supports the fact that up-regulation of other IGFBPs and the functional redundancy in this family might compensate for the lack of IGFBP-2 [van Kleffens et al., 1998; Wood et al., 2000; Wolf et al., 2000; Chesik et al., 2007]. Because other IGFBPs possibly compensate for the absence of IGFBP-2, it is possible that specific effects of IGFBP-2 cannot be identified in an IGFBP-2 knockout model. After all, the effect of an IGFBP-2 knockout is diminished if other IGFBPs take over its role.

2.3 Phenotypic alterations in transgenic mice overexpressing IGFBP-2

In order to investigate the effects of excess IGFBP-2, transgenic mice were generated that overexpressed IGFBP-2 [Hoeflich et al., 1999]. All organs except the liver displayed transgene expression. A threefold increase in serum IGFBP-2 levels was detected when compared to wild-type mice. Only a moderate reduction of approximately 8% in organ weights was found in these transgenic mice but a significant weight reduction was only seen in the spleen. Also, carcass weight was reduced by approximately 13%. Body weight was reduced by 10-13% in adult transgenic mice and this was mainly due to a reduce in carcass weight which accounts for 40-45% of total body weight. No body weight reduction was seen at birth, although IGFBP-2 transgene expression was already detected [Hoeflich et al., 1999]. Opposite phenotypic changes are seen in IGF-1 transgenic mice [Hoeflich et al., 1999; Schneider et al., 2000]. It is known that IGF-1 is a regulator of postnatal growth whereas IGF-2 is important for fetal growth. Because IGF-1 and IGF-2 serum levels were not affected, IGFBP-2 probably reduces IGF-1 bioavailability and therefore represents a negative regulator of postnatal growth [Hoeflich et al., 1999; Chesik et al., 2007].

Chapter 3: IGFBP-2 in tumorigenesis

3.1 Effects of IGFBP-2 in tumorigenesis

Several studies indicate that IGFBP-2 might somehow function as a promoter of cancer cell proliferation, migration and invasion. A positive association between elevated serum levels of IGFBP-2 and cell proliferation has been detected in colorectal cancers [El Atiq et al., 1994], ovarian tumors [Karasik et al., 1994; Flyvbjerg et al., 1997; Baron-Hay et al., 2004], prostate cancers [Cohen et al., 1993; Ho et al., 1997; Degraff et al., 2009], adrenocortical tumors [Boulle et al., 1998], breast cancers [Busund et al., 2005; So et al., 2008], gliomas [Fuller et al., 1999; Song et al., 2003; Wang et al., 2003] and leukemias [Mohnike et al., 1996]. Furthermore, a correlation has been detected between IGFBP-2 expression and tumor grade in various tumors such as colorectal [Mishra et al., 1998], adrenocortical [Boulle et al., 1998] and mammary tumors [McGuire et al., 1994; Korc-Grodzickiet al., 1996]. These clinical findings are also supported by in vitro studies showing that overexpression of IGFBP-2 has a tumorigenic potential. For example, a positive correlation was seen between IGFBP-2 mRNA expression and proliferation in cells of the human colon carcinoma cell line (Caco-2). Another model studied the effects of elevated IGFBP-2 on mouse adrenocortical tumor cells (Y-1 cells). These Y-1 cells did not express IGFBP-2 but an increase in tumorigenic potential was detected when Y-1 cells were transfected with an excess of IGFBP-2 [Hoeflich et al., 2001]. Targeting the expression of IGFBP-2 may be of value in treating some malignancies in the future.

In contrast with these studies, however, several others indicate that IGFBP-2 can inhibit cancer cell proliferation. In IGF-dependent cell culture systems, the addition of exogenous IGFBP-2 resulted in inhibition of cell proliferation. Furthermore, IGF analogues with reduced affinity for IGFBP-2 had a higher potency in stimulating cell proliferation than normal IGF-1 in different cell lines [Hoeflich et al., 2001]. For example, in C6 glioblastoma cells with low IGF levels, overexpression of IGFBP-2 resulted in reduced cell proliferation. The same result was also seen in colon carcinoma cells [Hoeflich et al., 1998; Martin et al., 2007].

Recently, several studies have identified potential mechanisms that could underlie the effect of IGFBP-2 in stimulating or inhibiting tumorigenesis and these mechanisms will now be discussed in further detail.

3.2 Potential stimulatory mechanisms of IGFBP-2 in tumorigenesis

Worldwide, breast cancer is the second most common type of cancer and IGFBP-2 overexpression is associated with breast carcinoma and invasive breast cancer. Perks et al. [2007] discovered a potential mechanism by which IGFBP-2 is involved in human breast cancer while they were studying the effect of IGF-2 on MCF-7 cells. They demonstrated that IGF-2 stimulates metabolic activity, proliferation and DNA synthesis up to a dose of 75-100 ng/ml in MCF-7 cells. However, higher doses of IGF-2 reduced the response. In contrast, a significantly greater response at a dose of 500 ng/ml was observed if an IGF-2 analog (Des(1-6)IGF2) with very little affinity for IGFBPs or a non-peptide ligand (NBI-31772) that displaces IGFs from all IGFBPs was used.

Because a previous study by Moorehead et al. demonstrated an IGF-2 feedback control of phosphatase and tensin homolog (PTEN) in mouse mammary glands, the response of PTEN

to exposure to IGF-2 was examined [Moorehead et al., 2003]. PTEN is a tumor suppressor gene that was one of the first phosphatases implicated in human cancers and is the second most frequently mutated gene in human cancers [Stambolic et al., 1998]. Such a mutation in PTEN can lead to tumor initiation, promotion of tumor progression and metastases. The function of PTEN is to dephosphorylate phosphatidylinositol triphosphates (PIP3) that are generated by the PI3K pathway and to suppress MAPK signaling [Weng et al., 2002; Mehrian-Shai et al., 2007]. Therefore, PTEN is able to oppose the proliferative activity of many growth factors by inhibiting the activation of downstream Akt that results in inhibition of cell survival and proliferation [Mehrian-Shai et al., 2007]. A tightly controlled feedback loop exists between PTEN and IGF-2 in which PTEN can downregulate IGF-2 expression and IGF-2 can enhance PTEN expression [Perks et al., 2007].

Perks et al. discovered that IGFBP-2 is able to regulate PTEN expression because MCF-7 cells treated with Des(1-6)IGF2 did not exhibit an increase in PTEN levels. In contrast, IGF-2 in MCF-7 cells did induce a marked increase in PTEN levels indicating that IGFBP-2 prevented the feedback increase in PTEN. This was also proven by the fact that in the presence of an IGFBP-2 blocking antibody, Des(1-6)IGF2 induced a similar increase in PTEN as IGF-2 did, indicating that IGFBP-2 probably uses the integrin receptor to exhibit its activity. So the difference in response to high concentrations of IGF-2 was associated with an increase in PTEN. IGFBP-2 did not prevent IGF-2 from binding to its receptor because no differences in PI3K and MAPK pathway activity were observed. Perks et al. therefore examined the levels of cyclin-dependant kinase inhibitor p21 for which it was already known that expression is regulated by PTEN [Wu et al., 2000]. Their findings indicated that an increase in IGFBP-2, not bound by IGF, blocks the IGF-2-induced feedback increase in PTEN that protects from overstimulation of IGF-2. This leads to an increase in p21 thereby stimulating the mitogenic response to IGF-2 in MCF-7 cells (Fig. 2). The balance between IGFBP-2 and PTEN may therefore play a crucial role in tumor progression [Perks et al., 2007].

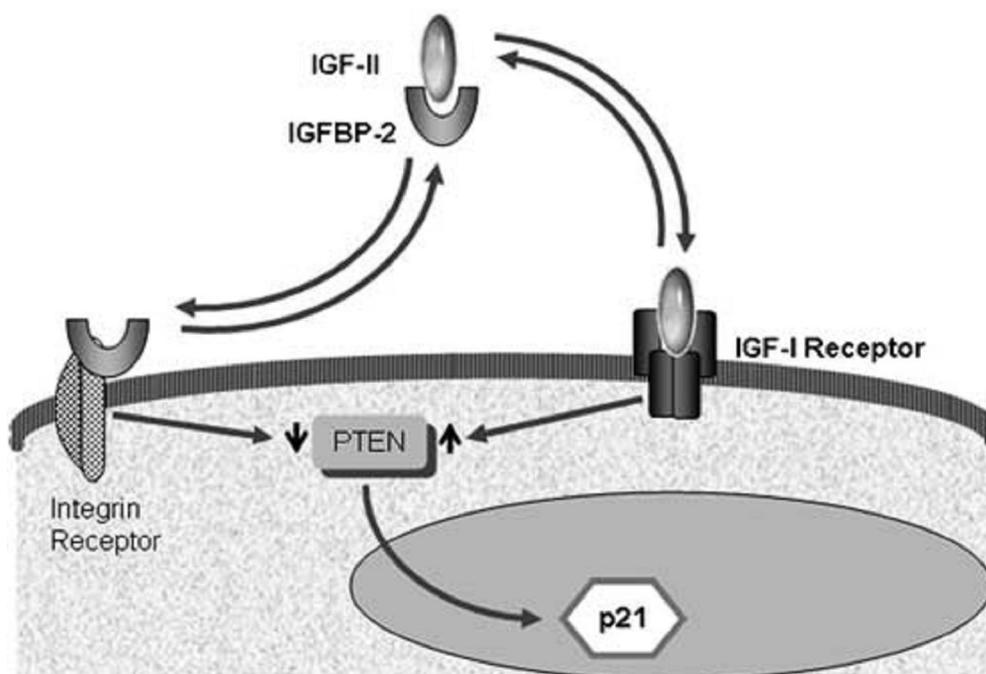


Figure 2. IGFBP-2 in human breast cancer cells. Schematic mechanism for IGFBP-2 and IGF-2 interactions via PTEN in MCF-7 breast cancer cells. (Source: Perks et al., 2007)

A loss of PTEN by increased levels of IGFBP-2 is also associated with glioblastomas, the most common and advanced type of primary brain tumor, and prostate cancer with poor prognosis. Serum IGFBP-2 levels may therefore be a potential biomarker for PTEN status in these two forms of cancer. IGFBP-2 overexpression was detected in approximately 50% of glioblastomas and is therefore associated with poor patient survival [Mehrian-Shai et al., 2007]. In vitro studies have already demonstrated that IGFBP-2 promotes glioma cell migration and invasion by the formation of a complex with integrin $\alpha 5$ and activation of matrix metalloproteinase 2 (MMP-2) [Wang et al., 2006; Dunlap et al., 2007].

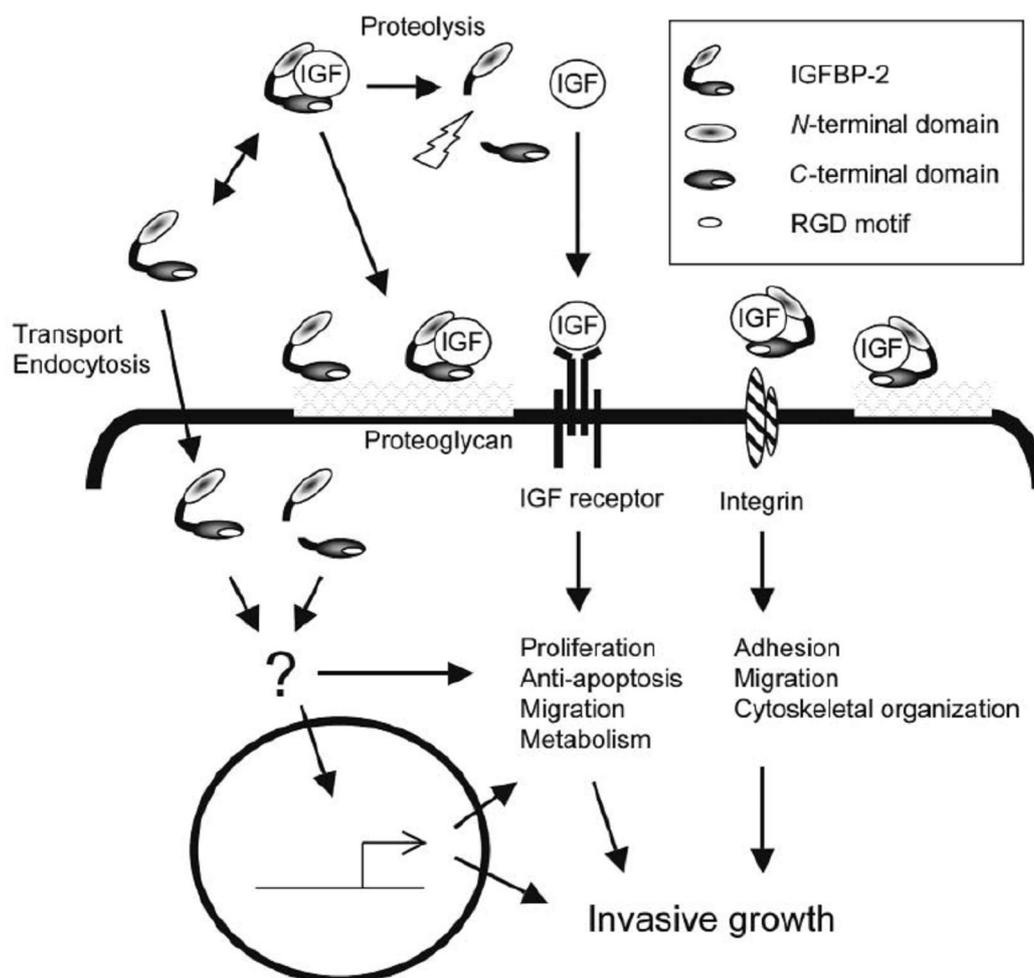


Figure 3. Proposed IGFBP-2-mediated mechanisms for invasive growth in glioblastoma cells. RGD-dependant cell surface association with integrin or RGD-independent binding to proteoglycan resulting in outside-in signaling. Proteolytic cleavage of IGFBP-2 resulting in decreased affinity for IGFs and therefore release of IGFs that induces IGF receptor-mediated signaling. Cytosolic and nuclear import of IGFBP-2 within glioblastoma cells results in additional IGF-independent functions. (Source: Fukushima et al., 2007)

Dunlap et al. [2007] studied whether IGFBP-2 also plays a driving role in glioma formation and progression. They used the mouse RCAS/*N-tva* somatic cell gene transfer model and discovered that IGFBP-2 alone was not sufficient for glioma development. However, IGFBP-2 in combination with V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (K-Ras), a GTPase involved in many signal transduction pathways, resulted in the formation of low-grade astrocytomas through activation of the Akt pathway. IGFBP-2 in combination with platelet-derived growth factor β (PDGFB) also led to the formation of high-grade oligodendrogliomas.

It was already known that PDGFB overexpression drives low-grade oligodendroglioma development in the RCAS/*N-tva* somatic cell gene transfer model [Dai et al., 2001]. Compared to low-grade oligodendrogliomas, these high-grade oligodendrogliomas as formed in mice with IGFBP-2 plus PDGFB expression, are characterized by increased cellular density, mitotic activity, vascular proliferation and necrosis.

It has been demonstrated that elevated levels of Akt and S6 kinase (S6K), a key protein kinase in the signaling pathway from Akt to the ribosome, are present in IGFBP-2-PDGFB-induced high-grade oligodendrogliomas but not in PDGFB-induced low-grade oligodendrogliomas. These results indicate that IGFBP-2 overexpression leads to glioma development and progression by significantly up-regulating and activating the Akt pathway when an additional oncogenic event (K-Ras or PDGFB) is present [Dunlap et al., 2007].

Fukushima et al. [2007] hypothesize that IGFBP-2 is crucial for invasive growth of glioblastoma cells because engineered overexpression of IGFBP-2 resulted in increased invasive capability and up-regulation of MMP-2 in glioblastoma cells. According to this result, knockdown of IGFBP-2 significantly reduced invasion. The exact mechanism remains somewhat unclear but some proposed IGFBP-2-mediated mechanisms include: 1) IGFBP-2/integrin-mediated outside-in signaling, 2) proteolytic cleavage of IGFBP-2 resulting in release of IGFs and IGF receptor-mediated signaling or 3) additional IGF-independent functions of IGFBP-2 after cytosolic and nuclear import (Fig. 3) [Fukushima et al., 2007]. However, the mechanism by which IGFBP-2 can be imported in the nucleus remains unclear because IGFBP-2 does not possess a classic nuclear localization signal [Miyajo et al., 2009].

Astrocytomas, the most common type of brain tumor in children, are also associated with an overexpression of IGFBP-2. Becher et al. [2008] compared the expression of approximately 30000 receptor tyrosine kinase- and receptor tyrosine kinase-associated genes in low-grade and high-grade astrocytomas to identify genes that are responsible for promoting the aggressive high-grade astrocytoma phenotype. IGFBP-2 was one of three genes that were significantly up-regulated in high-grade astrocytomas compared to low-grade astrocytomas. This indicates that IGFBP-2 can possibly play an important role in cell migration and astrocytoma invasion. Previous studies already reported an influence of the IGF system in astrocytoma invasion and recently it was shown that an interaction between the RGD domain of IGFBP-2 and the integrin $\alpha 5$ subunit promotes IGFBP-2 induced cell mobility in astrocytomas [Brockmann et al., 2003; Wang et al., 2003; Wang et al., 2006]. Becher et al. investigated the role of IGFBP-2 in the migration of astrocytoma cells. Therefore, they tested whether IGFBP-2 could play a role in promoting the high-grade astrocytoma phenotype by modulating astrocytoma gene expression. They stimulated the T98 and U87 astrocytoma cell lines with IGFBP-2, independent of IGF, and this resulted in the induction of DNA-dependant protein kinase catalytic subunit expression (DNA-PKcs), a nuclear serine/threonine kinase DNA repair enzyme that rejoins double-strand breaks. Because this was an unexpected outcome, Becher et al. analyzed astrocytoma cells and consistent with their previous outcome they found that DNA-PKcs was significantly up-regulated in high-grade astrocytomas compared to low-grade astrocytomas. DNA-PKcs activation in astrocytoma leads to resistance against radiation-induced and chemotherapy-induced damage. Thus, IGFBP-2 may protect astrocytomas from therapeutic killing by promoting DNA-PKcs expression [Becher et al., 2008].

Regulation and expression of IGFBP-2 is independent of the IGF system because IGF-1 is downregulated in high-grade astrocytomas. Therefore, it appears that the IGF-system is

dysregulated in astrocytomas because in normal neurodevelopment, IGFBP-2 and IGF-1 expression is temporally synchronized [Becher et al., 2008].

Miyako et al. [2009] discovered another mechanism by which IGFBP-2 is able to stimulate prostate carcinogenesis. Previous studies already demonstrated that IGFBP-2 can be isolated in the nucleus but the intranuclear role remained unclear. To elucidate a potential nuclear role, Miyako et al. used a human prostate cDNA library to identify binding partners of IGFBP-2. An interaction between IGFBP-2 and Pim-1-associated protein-1-associated protein-1 (PAPA-1) was found which was not specific for the nucleus. IGFBP-2 was present throughout the cell and PAPA-1 was mainly localized in the nucleus and co-localization was shown in the nuclei. PAPA-1 inhibits cell growth by causing cell cycle arrest at the G1-phase when localized in the nucleus [Kuroda et al., 2004]. PIM-1, a proto-oncogene used as prognostic marker in prostate cancer, activates the nuclear binding protein of PAPA-1 suggesting a link with cancer carcinogenesis. If the expression of PAPA-1 on mRNA and protein level was suppressed by using small interfering RNA (siRNA) against PAPA-1, this enhances IGFBP-2-induced cell proliferation indicating that PAPA-1 has a growth-inhibitory effect. This effect was also confirmed in mouse embryonic fibroblasts derived from an IGFBP-2 knockout mouse, indicating that IGFBP-2 is able to abrogate this growth-inhibitory effect.

Up-regulated expression of IGFBP-2 can also be seen in menin-null cells of patients with multiple endocrine neoplasia type I (MEN1). MEN1 is an autosomal dominant inherited cancer syndrome characterized by the development of tumors in several endocrine organs [Pannett et al., 1999]. *Men1* encodes for the nuclear protein menin and is the mutated gene in MEN1 patients. Menin can interact with multiple transcription factors and inhibits their activity [Chandrasekharappa et al., 1997]. La et al. [2004] identified genes that were repressed by menin expression and IGFBP-2 was one of them. A 14-fold reduction of IGFBP-2 expression was detected in cells expressing menin compared with menin-null cells. Results indicated that menin has a critical role in repressing IGFBP-2 expression. Wild-type menin reduces the expression of IGFBP-2 by inhibiting the promoter of IGFBP-2. In more detail, menin alters the promoter structure of the IGFBP-2 gene. Two independent nuclear localization signals (NLSs) are responsible for the localization of menin in the nucleus. Repression of IGFBP-2 expression is compromised when these NLSs in menin are mutated. However, a single NLS is enough for targeting menin to the nucleus but both NLSs play a crucial role in repressing IGFBP-2. In conclusion, menin represses the expression of IGFBP-2 but a mutation in both menin or NLS can compromise the role of menin in repressing IGFBP-2 expression [La et al., 2004].

3.3 Potential inhibitory mechanisms of IGFBP-2 in tumorigenesis

In recent years, several studies have suggested a potential inhibitory role of IGFBP-2 in tumorigenesis besides these previously mentioned stimulatory mechanisms. One of these inhibitory mechanisms has been demonstrated by Grimberg et al. [2006]. They identified IGFBP-2 as one of the targets for p53-mediated transcriptional activation. Further examination revealed eight intronic and promoter sites with high homology to the p53 consensus sequence.

P53 is an important tumor suppressor encoded by TP53 which is the most frequently mutated gene in human cancers [Guimaraes et al., 2002]. P53 plays a critical role in cell cycle

checkpoints that become activated by DNA damage, oncogenic stress or hypoxia and therefore induce cell cycle arrest and apoptosis [Giono et al., 2006]. Because p53 has many transcriptional targets, Grimberg et al. knocked-down IGFBP-2 expression to determine the impact of IGFBP-2 regulation by p53. This resulted in a significant increase in activated ERK in cells exposed to IGF-1 due to a blockade in p53-mediated suppression of phosphorylated ERK expression (Fig. 4). This indicates the important role of IGFBP-2 as a transcriptional target of p53 because IGFBP-2 binds IGF-1, thereby preventing IGF-1R stimulation. Another effect of p53 on the IGF system is decreasing IGF/IGF-1R signaling by inhibiting DNA binding to the initiator region of IGF-1R gene and to the third promoter of the IGF-2 gene [Grimberg et al., 2006].

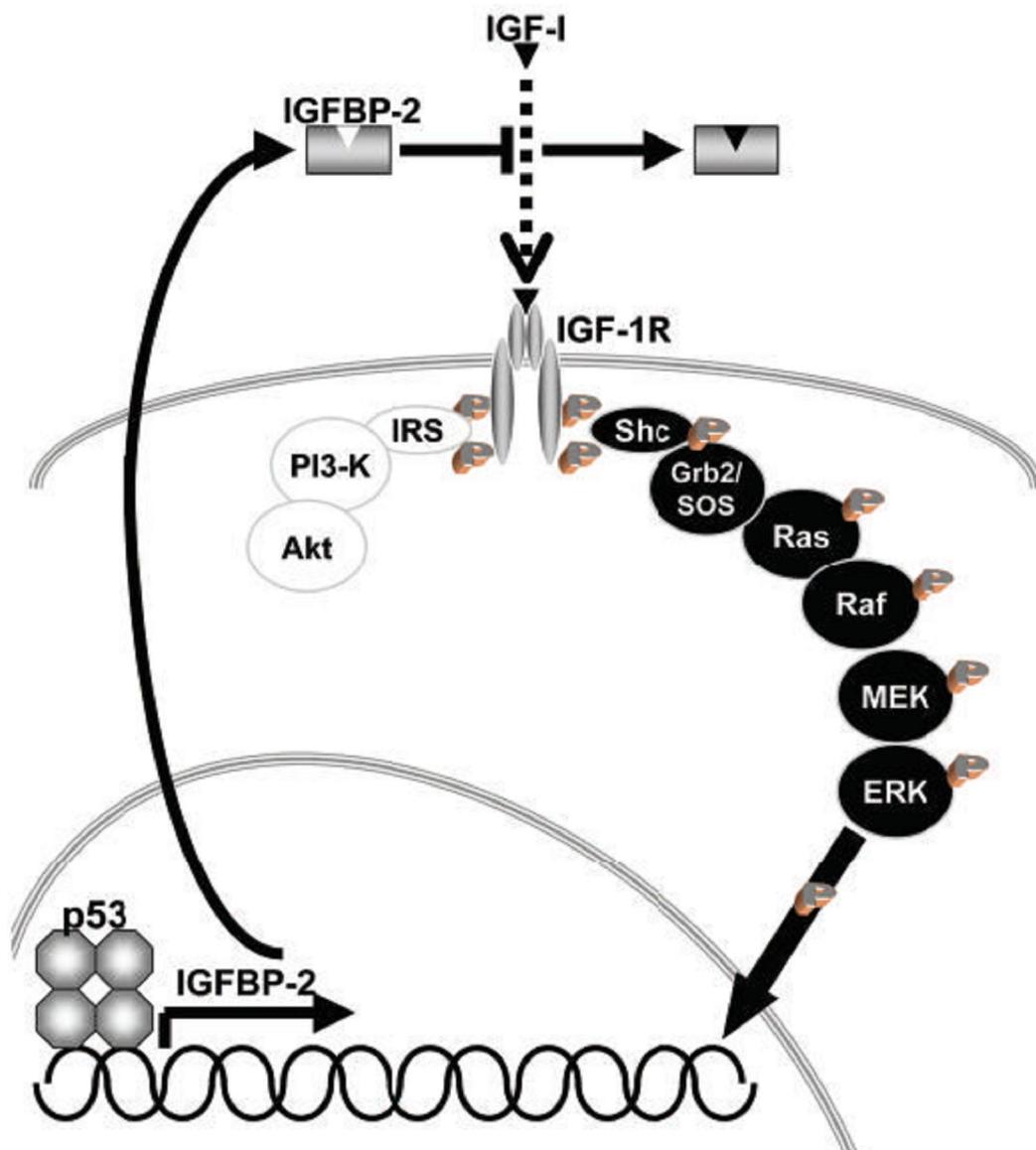


Figure 4. Model of p53 effects on the IGF system. P53 stimulates the transcription of IGFBP-2 that competes with IGF-1R to bind IGF-1, thereby preventing IGF-1R stimulation and activation of the PI3K and MAPK pathways, leading to cell survival and mitogenesis. IGFBP-2 knock-down in vitro prevented p53-mediated suppression of ERK activation by IGF-1 signaling. (Source: Grimberg et al., 2006)

We previously mentioned that IGFBP-2 had a positive effect on tumorigenesis in astrocytomas by inducing cell mobility and protecting astrocytoma cells against therapeutic

killing. However, Rorive et al. [2008] showed that IGFBP-2 is also able to inhibit cell growth and motility in astrocytomas. The aggressiveness of astrocytomas mainly depends on the growth rate of the tumor cells and their ability to infiltrate in the brain parenchyma [Kleihues et al., 2002]. MMPs have been reported to play an important role in the interaction between astrocytoma cells and the ECM, crucial for cell growth and migration [Rao, 2003]. High levels of MMP expression is associated with astrocytoma malignancy and invasion [Nuttall et al., 2003]. Rorive et al. demonstrated that MMP-2, MMP-9 and MT1-MMP were able to cleave IGFBP-2, the most widely expressed IGFBP in the CNS, and therefore the IGFBP-2-IGF-2 complex [Thraillkill et al., 1995; Claussen et al., 1997; Menouny et al., 1997; Coppock et al., 2004; Nakamura et al., 2005]. They used a stable IGFBP-2-IGF-2 complex and it turned out that only MMP-9 was able to cleave IGFBP-2, resulting in the release of free IGF-2 that promotes cell growth and migration. In human LN229 high-grade astrocytoma cells, IGFBP-2 alone had no influence on cell growth. The formation of an IGFBP-2-IGF-2 complex inhibited LN229 cell growth but the addition of MMP-9 significantly increased cell growth due to the proteolysis of the IGFBP-2-IGF-2 complex. This indicates that MMPs are not only able to destroy the ECM which is associated with tumor cell invasion but they can also cleave non-ECM-substrates to alter tumor cell behavior [Rorive et al., 2008].

So IGFBP-2 acts as a modulator of IGF-2 actions in the CNS and the IGFBP-2-IGF-2 complex inhibits astrocytoma cell growth. In addition to this result, the IGFBP-2-IGF-2 complex may also prevent the interaction between the RGD domain of IGFBP-2 and the integrin $\alpha 5$ subunit that promotes IGFBP-2 induced cell mobility in astrocytomas.

Another mechanism by which IGFBP-2 is able to inhibit cell growth is identified by Pereira et al. [2004] and depends on the interaction of IGFBP-2 with $\alpha\beta 3$ integrin. This type of integrin was already been demonstrated to be expressed at low levels in normal tissue and increased expression was seen in several types of cancer [Vonlaufen et al., 2001; Cooper et al., 20002; Ria et al., 2002; Sturm et al., 2002]. The $\alpha\beta 3$ integrin receptor is probably a different type of integrin receptor than the one indicated by Perks et al. [2007] because IGFBP-2 used that receptor to stimulate human breast cell growth.

Direct association of $\alpha\beta 3$ integrin with growth factor receptors, including those of the IGF system, enhances tumor cell progression [Giancotti et al., 1999]. Binding of $\alpha\beta 3$ integrin to vitronectin enhances IGF-1 mediated proliferation and migration. Therefore, it could be important to block this interaction, thereby reducing IGF-1 signaling. Pereira et al. used the MCF-7 human breast cancer cell line with a high expression of $\alpha\beta 3$ integrin. They found a significant decrease in MCF-7 cell migration toward IGF-1 and IGF-2 when $\alpha\beta 3$ integrin was expressed. This migration profile was reversed when performed on vitronectin suggesting that a negative regulatory signal competes with vitronectin to bind $\alpha\beta 3$ integrin. When Pereira et al. identified this negative regulatory signal it turned out that IGFBP-2 was responsible for the $\alpha\beta 3$ integrin inhibition of IGF-mediated migration. Cell-associated IGFBP-2 was significantly decreased in cells cultured on vitronectin suggesting that vitronectin can displace IGFBP-2 from the cell surface [Pereira et al., 2004].

A mouse model was used to test whether IGFBP-2 and $\alpha\beta 3$ integrin also interact in vivo and influence tumor growth. Pereira et al. demonstrated that IGFBP-2 and $\alpha\beta 3$ integrin indeed do interact in vivo and this was associated with reduced tumor growth due to an abrogation in IGF-1 and IGF-2-mediated migration and proliferation. Two possible mechanisms may explain why IGFBP-2 is able to influence IGF action. IGFBP-2 may sequester with the cell surface by $\alpha\beta 3$ integrin and compete with IGF-Rs to bind free IGF-1 and IGF-2. The second

possibility is that IGFBP-2 binding to $\alpha\beta3$ integrin influences integrin-mediated signaling to regulate IGF activity. A reduction in $\alpha\beta3$ integrin has been demonstrated in breast tumors compared to benign tumors indicating that a down-regulation of $\alpha\beta3$ integrin is used to overcome the suppressive effect on the IGF system [Pereira et al., 2004].

Chapter 4: IGFBP-2 in metabolic homeostasis

4.1 Effects of IGFBP-2 in metabolic homeostasis

Besides IGFBP-2's role in tumorigenesis, there also seems to be an involvement in metabolic homeostasis. Although insulin is the primary regulator of blood glucose levels, accumulating evidence indicates that IGF-1 also plays an important role [Wheatcroft et al., 2009]. This connection between the IGF-1 and insulin seems logical because they share a high degree of homology and both coordinate energy uptake and growth through the PI3K pathway [Nakae et al., 2000]. IGF-1 can directly stimulate glucose uptake in target cells and indirectly increase sensitivity of tissues to insulin. However, insulin is able to regulate IGF-1 bioactivity by modulating the six IGFBPs that each exert different actions on the bioactivity of IGF-1 [Lewitt et al., 1991; Yakar et al., 2001; Wheatcroft et al., 2009; Arafat et al., 2009]. It was already known that IGFBP-1 positively correlates with insulin sensitivity [Maddux et al., 2006] but recently IGFBP-2 has emerged as another potential player [Van Haeften et al., 2007; Arafat et al., 2009]. In vitro studies do not explicitly show whether IGFBP-2 is positively regulated by insulin [Boni-Schnetzler et al., 1990; Schmid et al., 1992; Bradshaw et al., 1993].

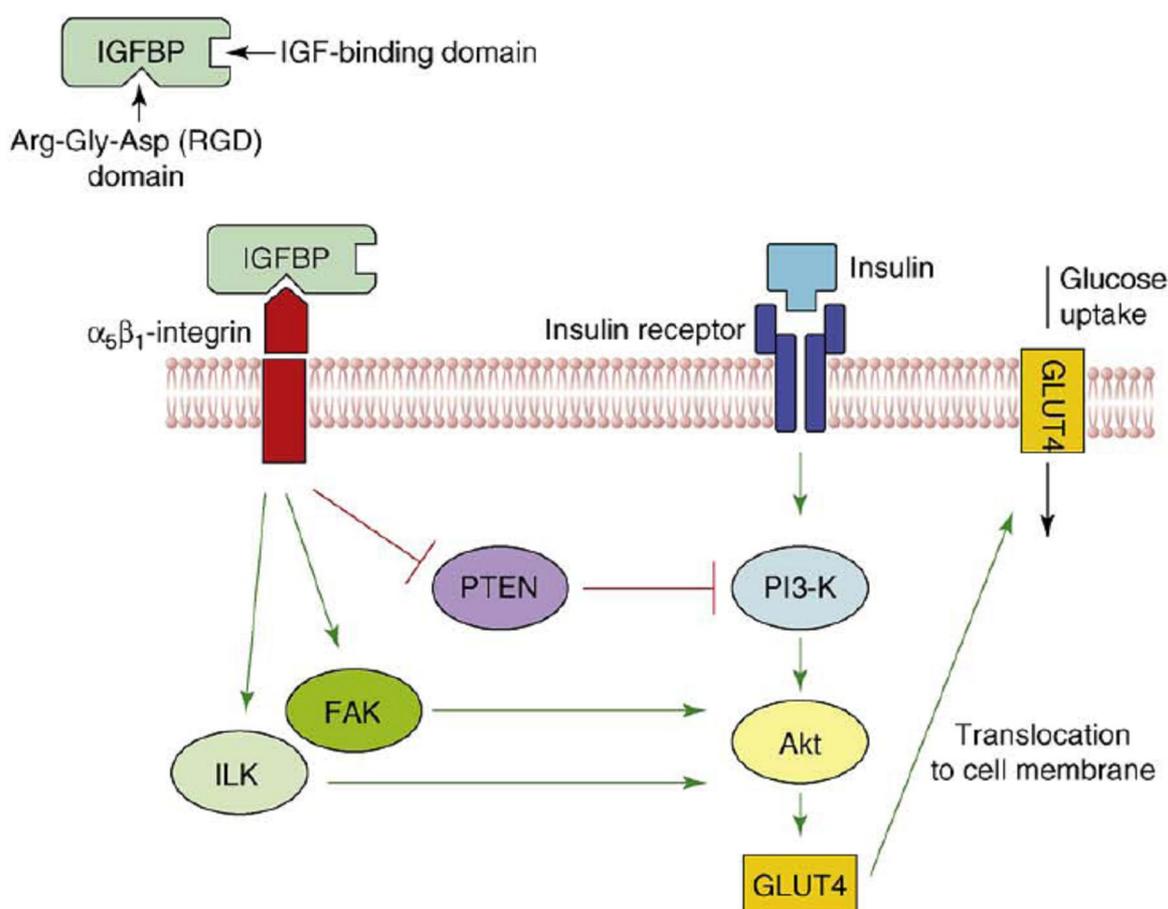


Figure 5. Presumable mechanisms by which IGFBP-2 modulates insulin sensitivity independent of IGF-1. The RGD sequence of IGFBP-2 binds $\alpha_5\beta_1$ -integrin on the cell surface in certain cell types. This integrin-receptor activation inhibits PTEN and activates FAK and ILK, thereby modulating insulin signaling via the PI3K pathway. (Source: Wheatcroft et al., 2009)

IGF-1 is able to bind the IGF receptor leading to insulin-like effects on glucose by activation of the PI3K pathway and ultimately to a translocation of glucose transporter 4 (GLUT-4) to the cell membrane in striated muscle and fat cells (Fig. 5). Because IGFBP-2 has a higher affinity for IGF-1 than IGF-Rs, IGFBP-2 is able to reduce free, available IGF-1 and thereby inhibits signaling via the IGF receptor [Frystyk et al., 2004; Wheatcroft et al., 2009]. The effect of IGFBP-2 on IGF-1 depends on whether it is unbound or associated with the ECM or cell surface [Wheatcroft et al., 2009].

IGFBP-2 independent of IGF-1 also has an effect on insulin sensitivity when it binds the $\alpha 5\beta 1$ -integrin via its RGD sequence. This binding of IGFBP-2 to $\alpha 5\beta 1$ -integrin results in the inhibition of PTEN, a negative regulator of insulin signaling in skeletal muscle and adipocytes, and the phosphorylation of focal adhesion kinase (FAK) and integrin-linked kinase (ILK). This ultimately results in an increase in PI3K activity and the translocation of GLUT-4 to the cell membrane (Fig. 5). So IGFBP-2 is able to modulate insulin sensitivity by altering PI3K activity via IGF-dependant and independent mechanisms [Wheatcroft et al., 2009].

4.2 Associations of IGFBP-2 with obesity

Because of lifestyle, obesity nowadays is an important health problem in developed countries. Obese people have a markedly lower life expectancy due to a higher risk of developing type 2 diabetes, insulin resistance, hypertension and cardiovascular disease [Fontaine et al., 2003]. It was already reported that IGF-1 plays a role in proliferation and differentiation of adipocytes, and that IGFBP-2 is secreted by preadipocytes during adipogenesis and IGFBP-2 serum concentrations are reduced in people with obesity [Smith et al., 1988; Boney et al., 1994; Frystyk et al., 1999]. Wheatcroft et al. [2007] therefore investigated the role of IGFBP-2 in modulating the effects of IGF-1 in obesity by using a transgenic mouse model that overexpresses the human IGFBP-2 gene under control of its native promoter. Compared with wild-type mice, these transgenic IGFBP-2 mice expressed a 2.2 fold higher serum IGFBP-2 level and no developmental abnormalities could be detected. Although insulin levels were similar, transgenic mice had significantly lower nonfasted blood glucose levels than controls at the age of 4-6 months. They used a glucose tolerance test (GTT) to determine how quickly glucose was cleared from the blood. It turned out that in control mice, glucose tolerance declined with age but this was not seen in transgenic mice. Ageing transgenic IGFBP-2 mice did not express an increase in plasma insulin concentrations so this could therefore not explain the prevention of age-induced insulin resistance, indicating that IGFBP-2 probably is responsible for the protection against age-induced insulin resistance [Wheatcroft et al., 2007].

To determine the effect of IGFBP-2 overexpression on the development of obesity, Wheatcroft et al. fed transgenic and control mice with standard chow or a high-fat diet for 32 weeks. The two groups of mice that were fed with standard chow showed a similar weight gain but weight gain in transgenic mice fed with a high-fat diet was significantly declined compared with wild-type mice. This weight gain in wild-type mice was due to an increase in their fat depots whereas the transgenic mice remained slim and displayed a reduction in fat cell size. Wheatcroft et al. also weighed all organs and only the liver weight was lower in transgenic mice fed with a high-fat diet. Insulin and glucose levels between both groups of mice were approximately the same on both types of diet. However, after a GTT it turned out that blood glucose levels in transgenic mice were significantly lower compared with wild-type mice on either diet. These results indicate that IGFBP-2 transgenic

mice are resistant to diet-induced obesity and fatty liver. Therefore, Wheatcroft et al. wanted to know whether IGFBP-2 itself was able to impair adipogenesis and they used 3T3-L1 preadipocytes to test this. These preadipocytes were induced to differentiate in the presence of human IGFBP-2. IGFBP-2 significantly reduced adipogenesis when preadipocytes were induced with a serum-containing induction cocktail that was supplemented with human IGF-1. In contrast, adipocytes could differentiate in the presence of high-dose insulin or Des(1-3)IGF-1 together with human IGFBP-2. These results demonstrate that IGFBP-2 inhibits adipogenesis by modulating IGF-1 activity [Wheatcroft et al., 2007]. Concluding, reduced susceptibility to obesity can be seen in transgenic mice that overexpress IGFBP-2. This is due to inhibition of IGF-1-mediated adipogenesis which is known to be increased when preadipocytes differentiate into mature adipocytes [Doglio et al., 1987].

4.3 Associations of IGFBP-2 with metabolic syndrome

Arafat et al. [2009] wanted to determine the modulation of IGFBP-2 by insulin in humans with metabolic syndrome. Metabolic syndrome is also known as insulin resistance syndrome and this is a condition in which normal insulin levels are inadequate to produce a normal insulin response in muscle, fat and liver cells. This results in reduced glucose uptake in muscle cells, elevated hydrolysis of stored triglycerides in fat cells and an elevated fatty acid concentration in blood plasma, and impaired glycogen synthesis and no suppression of glucose production in liver cells. All these factors contribute to an elevation of blood glucose levels. Arafat et al. compared results between healthy persons and persons with impaired glucose tolerance (IGT), which is associated with insulin resistance, and found that persons with IGT had lower IGFBP-2 concentrations. Furthermore, these persons had significantly higher BMI, triglycerides, fasting glucose and fasting insulin levels which all corresponds with metabolic syndrome. They also found that under euglycemic conditions in healthy persons, insulin suppresses IGF-1 bioactivity by increasing IGFBP-2 concentrations. However, this suppression of IGF-1 bioactivity was not detected in IGT persons probably due to a reduction in IGFBP-2 level. Thus, a positive correlation could be seen between IGFBP-2 levels and insulin sensitivity, thereby indicating that the reduction of IGFBP-2 in IGT persons was due to resistance to insulin actions [Arafat et al., 2009]. This assumption is in accordance with previous studies which demonstrate that an increase in IGFBP-2 concentrations improves insulin sensitivity in obese subjects [Rasmussen et al., 2006; Wheatcroft et al. 2007].

Heald et al. [2006] used a cohort of 163 patients with type 2 diabetes and divided these subjects in two groups, namely presence or absence of metabolic syndrome. IGFBP-2 levels in patients with metabolic syndrome turned out to be lower compared to IGFBP-2 levels of patients without metabolic syndrome. With this study, they demonstrated that IGFBP-2 can also be used as a marker for metabolic syndrome [Heald et al., 2006].

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